# Evaluation in vitro of the antagonistic substances produced by *Lactobacillus* spp. isolated from chickens

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## Abstract

To determine the inhibitory capacity of lactic acid bacteria due to the action of antagonistic substances, we tested 474 isolates of *Lactobacillus* from the crop and cecum of chickens against gram-positive and gram-negative indicator microorganisms by the spot-on-the-lawn and well-diffusion antagonism methods. Of the 474 isolates, 265 demonstrated antimicrobial activity against the indicator microorganisms. Isolates identified as *L. reuteri*, *L. salivarius*, or *Lactobacillus* spp. inhibited *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes*, and *Salmonella* spp. but not *L. casei*, *L. delbrueckii*, *L. fermentum*, or *L. helveticus* by the well-diffusion simultaneous antagonism method under anaerobic incubation conditions. The antagonistic substances produced by some of the *Lactobacillus* isolates were inactivated after treatment by proteolytic enzymes, which suggested that the substances could be antimicrobial peptides or bacteriocins.

## Résumé

*Un total de 474 souches de bactéries lactiques appartenant au genre* Lactobacillus *ont été isolées du jabot et du caecum de poulets. Afin de déterminer la capacité inhibitrice de ces souches associée à l'action de substances antagonistes, l'activité antimicrobienne a été testée envers des microorganismes indicateurs à gram positif et à gram négatif par une méthode de diffusion en puit ainsi que par une méthode de plaque sur tapis bactérien. Parmi les 474 souches, 265 montraient une activité antimicrobienne envers les microorganismes indicateurs. Les souches identifiées comme étant* L. reuteri, L. salivarius, *ou* Lactobacillus *spp. ont inhibé* Enterococcus faecalis, E. faecium, Listeria monocytogenes, *et* Salmonella *spp. mais pas* L. casei, L. delbrueckii, L. fermentum, *ou* L. helveticus *par l'épreuve de diffusion simultanée en puit effectuée dans des conditions d'incubation en anaérobiose. Les substances antimicrobiennes produites par certaines souches de* Lactobacillus *étaient inactivées suite à un traitement par des enzymes protéolytiques, suggérant ainsi que ces substances pourraient être des peptides antimicrobiens ou des bactériocines.*

*(Traduit par Docteur Serge Messier)*

## Introduction

The lack of more effective methods to control avian salmonellosis and the indiscriminate use of antibiotics throughout the life of the animal have led the scientific community to search for an alternative to antibiotics in the production of chickens that does not damage the normal intestinal microflora or leave residues in the animal carcass (1). The use of probiotics in the avian diet contributes to health and growth since it generates a stable intestinal ecosystem, impairing colonization by pathogenic bacteria (2,3). The selection of bacteria such as *Lactobacillus*, *Pediococcus*, *Bacteroides*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, and *Escherichia coli* for use as probiotics is based on assessment of their metabolic products and their potential to colonize specific sites (4).

*Lactobacillus reuteri*, a normal inhabitant of the gastrointestinal tract of humans and animals, can synthesize and secrete antimicrobial substances of proteic origin that have antagonistic actions against gram-positive and gram-negative bacteria, yeast, fungi, protozoa, and viruses (5). Lindgren and Dobrogosz (6) reviewed the antagonistic activity of lactic acid bacteria against pathogens and spoilage bacteria. The mechanisms involve the production of lactic and acetic acids, nutrient depletion, hydrogen peroxide production, changes in oxidation/reduction potential, and production of antibiotic-like compounds (7). Many studies have dealt with the preservation of meat products by means of starters of lactic acid bacteria, and interest has been focused on strains producing bacteriocins (8,9).

Bacteriocins derived from lactic acid bacteria, usually small, heterogeneous, cationic proteins consisting of 30 to 60 amino acid residues, show marked variation in action spectrum, molecular weight, and biochemical properties (10,11). Currently, the best-characterized bacteriocin is nisin, which is produced by some strains of *Lactococcus lactis* subsp. *lactis*. Nisin is the only bacteriocin internationally approved and legalized for use in food (12). Many other bacteriocins produced by lactic acid bacteria are still being characterized (13).

Our study aimed to isolate and identify lactic acid bacteria of the genus *Lactobacillus* originating from the crop and cecum of chickens

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and to determine, by the spot-on-the-lawn and well-diffusion antagonistic methods, the inhibitory capacity of substances produced by these bacteria against gram-positive and gram-negative indicator microorganisms.

# Materials and methods

## Isolation and identification of *Lactobacillus*

The crop and cecum of 6 breeder chickens of Cobb lineage aged 52 wk and 6 of Ross lineage aged 65 wk were aseptically removed and transferred individually to tubes containing 10 mL of DeMan–Rugosa–Sharpe (MRS) broth (Oxoid Brasil, São Paulo, Brazil), pH 6.5. The tubes were incubated at 37°C for 48 h under anaerobic conditions, with the Gas Pak (Becton Dickinson and Company — Brasil, São Paulo, Brazil) disposable gas generator method. The cultures were then seeded onto MRS agar for growth and isolation of colonies. Round, white colonies with well-delineated borders and a creamy aspect, 2 to 3 mm in diameter, were submitted on Petri dishes for bacterial identification.

Identification of the genus *Lactobacillus* was based on morphologic and physiologic characteristics determined by Gram-staining, catalase and potassium hydroxide tests, gas production on glucose, according to the method of Collins and Hartlein (14), and fermentation of carbohydrates (arabinose, fructose, galactose, glucose, mannitol, mannose, maltose, sucrose, salicyl, and sorbitol), as described by Kandler and Weiss (15). Species were identified by multiplex polymerase chain reaction (PCR) with the use of primer initiators for *L. reuteri*, *L. fermentum*, and *L. salivarius* with oligonucleotide sequences described by Song et al (16).

## Determination of antimicrobial activity in vitro

The inhibitory activity against indicator microorganisms (Table I) of all 474 isolates of *Lactobacillus* spp. was determined by the spot-on-the-lawn antagonism method, as modified by Harris et al (17), and the well-diffusion simultaneous antagonism method, as described by Lewus and Montville (18).

*Spot-on-the-lawn antagonism method* — The 474 *Lactobacillus* isolates were seeded in point (20-μL) form onto MRS agar plates and incubated at 37°C for 12 h under aerobic conditions. Culture medium containing the indicator microorganisms was transferred to tubes containing 5 mL of MRS broth for lactic acid bacteria (*Lactobacillus* and *Enterococcus*) and 5 mL of brain–heart infusion (BHI) broth (Oxoid) for the other bacteria (*Listeria monocytogenes* and *Salmonella* serotypes) and incubated at 37°C for 12 h under aerobic conditions. Then 200 μL of the suspension was transferred to 20 mL of MRS broth for lactic acid bacteria and 20 mL of BHI broth for the other bacteria. The mixture was supplemented with previously prepared 0.75% agar-agar and maintained in a water bath at 45°C. Each indicator culture was poured onto the plates cultured with *Lactobacillus*. After complete solidification of the upper layer, the plates were incubated for an additional 24 h at 37°C under aerobic conditions. Of the 474 *Lactobacillus* isolates, 265 showed antimicrobial activity against the indicator microorganisms, observed as the formation of inhibition zones, and were chosen for the well-diffusion simultaneous antagonism method.

**Table I. Inhibition of indicator microorganisms by *Lactobacillus* isolates originating from the crop and cecum of 12 chickens, as determined by the spot-on-the-lawn and well-diffusion antagonism methods**

| | Antagonistic method; no. of sensitive strains (total no. tested) | |
|---|---|---|
| Indicator microorganism | Spot-on-the-lawn ($n$ = 265) | Well diffusion ($n$ = 53) |
| *Enterococcus faecalis* 19433 | 167 | 33 |
| *E. faecium* 19434 | 171 | 3 |
| *L. casei* 393 | 133 | 0 |
| *L. helveticus* 15009 | 119 | 0 |
| *L. fermentum* 14931 | 95 | 0 |
| *L. delbrueckii* subsp. *lactis* 4797 | 115 | 0 |
| *Listeria monocytogenes* 19115 | 169 | 34 |
| *Salmonella* Enteritidis 13076 (phagotype 4) | 192 | 10 |
| *S.* Enteritidis 6961 (phagotype 28) | 176 | 14 |
| *S.* Typhimurium 14028 | 174 | 11 |
| *S.* Pullorum 9120 | 163 | 2 |

*Well-diffusion simultaneous antagonism method* — Cell-free supernatants of the 265 *Lactobacillus* isolates showing antimicrobial activity against the indicator microorganisms by the spot-on-the-lawn method were obtained by centrifugation at 7500 × $g$ for 10 min in MRS broth supplemented with 0.05% glucose and incubated for 18 h at 37°C under aerobic conditions. Next, the pH was adjusted to 6 with 10 N NaOH, and the samples were filtered through a Millex-GV microfilter membrane with a pore size of 0.22 μm and a diameter of 25 mm (Millipore [Brasil], São Paulo, Brazil). The supernatant samples were stored at 8°C. Aliquots (20 μL) of the indicator cultures were transferred to 20 mL of Hektoen broth (Oxoid) supplemented with 0.75% agar-agar (*Salmonella* serotypes), MRS broth supplemented with 0.75% agar-agar (*Lactobacillus* and *Enterococcus*), and BHI broth supplemented with 0.75% agar-agar (*L. monocytogenes*). The broths were then poured onto Petri dishes. After complete solidification, 6-mm wells were punched, and 60 μL of the cell-free supernatant was placed in each well. The indicator cultures had an optical density of 0.102 to 600 nm, which corresponded to approximately $10^6$ colony-forming units per milliliter. The plates were incubated at 37°C for 24 h under anaerobic conditions. Of the 265 *Lactobacillus* isolates, 53 again showed antimicrobial activity against the indicator microorganisms, observed as formation of an inhibition zone around the wells.

## Detection of bacteriophages

The sandwich method, modified according to Hechard et al (13), was used to detect bacteriocin-producing *Lactobacillus* strains. The 53 isolates of *Lactobacillus* that showed antimicrobial activity against the indicator microorganisms by the well-diffusion method were seeded in point form onto Petri dishes containing MRS agar and incubated at 37°C for 12 h under aerobic conditions. A layer of nutrient broth supplemented with 1.5% agar-agar was added and left to

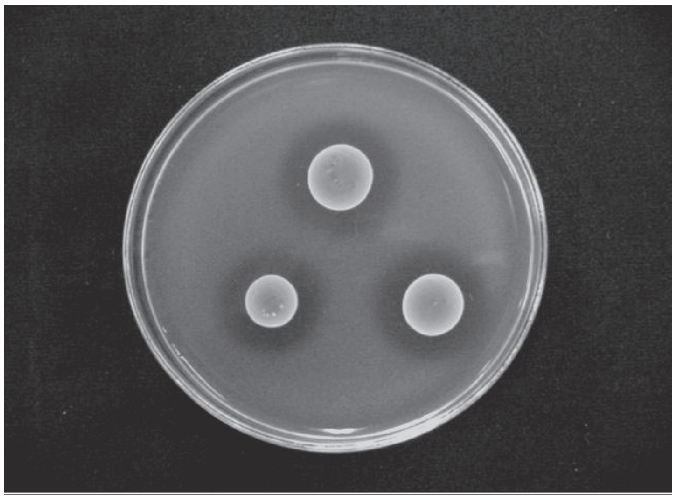

**Figure 1. Antimicrobial activity of *Lactobacillus reuteri* against *Salmonella* Enteritidis phagotype 4 as demonstrated by the inhibition zones produced with the spot-on-the-lawn antagonism method.**

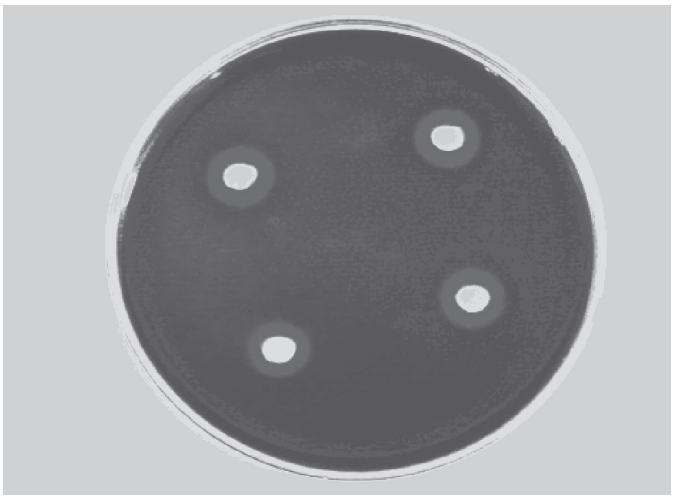

**Figure 2. Antimicrobial activity of *L. reuteri* against *Salmonella* Enteritidis phagotype 4 as demonstrated by the inhibition zones produced with the well-diffusion antagonism method.**

solidify, and then the BHI broth supplemented with 0.75% agar-agar containing indicator microorganisms was added. The plates were again incubated at 37°C for 24 h under anaerobic conditions. The action of antagonistic substances against the indicator microorganisms was demonstrated by the formation of inhibition zones around the wells. Lack of inhibition would indicate the action of bacteriophages against the indicator microorganisms due to diffusion.

## Sensitivity of antagonistic substances to enzymes

The sensitivity of antimicrobial substances to enzymes was assayed according to the method of Bromberg et al (19). Cell-free supernatants from the 53 *Lactobacillus* isolates that showed antimicrobial activity against the indicator microorganism by the well-diffusion method were collected by centrifugation (at 7500 × *g* for 10 min at 4°C) of MRS broth cultures that had been maintained overnight. The pH of the supernatants was adjusted to 6 with 10 N NaOH and treated with the following enzymes (distributed by Sigma-Aldrich Brasil, São Paulo, Brazil) at a final concentration of 0.2 mg/mL: pronase E in 20 mM of Tris-HCl, pH 7.8; α-chymotrypsin in 20 mM of Tris-HCl, pH 8.0; trypsin in 40 mM of Tris-HCl, pH 8.2; and pepsin in 0.002 N HCl, pH 6.0. The enzyme solutions were filter-sterilized through a Millex-GV microfilter membrane with a pore size of 0.22 μm and a diameter of 25 mm and then added to the sterile cell-free supernatants (1/1 v/v). Controls consisted of solutions of cell-free supernatant in 0.1 M sodium phosphate buffer without enzymes. Sensitivity was defined as the reciprocal of the highest dilution causing inhibition of the indicator strain multiplied by 100 to express the results as activity units per milliliter.

## Statistical analysis

The mean and standard deviation of the diameter of the inhibition zone for the indicator microorganisms that were determined to be sensitive to *L. reuteri*, *L. salivarius*, and *Lactobacillus* spp. (*E. faecalis*, *E. faecium*, *L. monocytogenes*, *Salmonella* Enteritidis phagotype 4, *Salmonella* Enteritidis phagotype 28, *Salmonella* Typhimurium, and *Salmonella* Pullorum) were compared by analysis of variance with the use of treatments in a factorial scheme for a completely randomized design (20).

# Results

The 474 bacterial isolates from the crop and cecum of the poultry were identified as *Lactobacillus* on the basis of a negative potassium hydroxide reaction and negative result of the catalase test and were characterized as gram-positive rods. Their carbohydrate-fermentation characteristics (production of gas from glucose) fit the heterofermentative and homofermentative groups of *Lactobacillus*. The 265 isolates that demonstrated antimicrobial activity against gram-positive and gram-negative indicator microorganisms with the spot-on-the-lawn antagonism method (Figure 1) were identified as *L. reuteri* (132), *L. salivarius* (45), or *Lactobacillus* spp. (88) by multiplex PCR. These isolates inhibited indicator microorganisms of the genera *Enterococcus*, *Listeria*, and *Salmonella* but showed no antagonistic activity against the *Lactobacillus* species *casei*, *delbrueckii*, *fermentum*, and *helveticus* by the well-diffusion simultaneous antagonism method (Table I).

Of these 265 *Lactobacillus* isolates, 53 (30 *L. reuteri*, 12 *L. salivarius*, and 11 *Lactobacillus* spp.) also showed antimicrobial activity against the same gram-positive and gram-negative indicator microorganisms with the well-diffusion antagonism method under anaerobic conditions (Table I). The halo of inhibition ranged in diameter from 1.0 to 6.0 mm (Figure 2). Analysis of variance of the mean diameters demonstrated that the *Salmonella* indicator microorganisms were significantly more sensitive than the *Enterococcus* indicator microorganisms to the antagonistic substances produced by *L. reuteri* and that *Lactobacillus* spp. had a significantly greater action than *L. reuteri* and *L. salivarius* on *Enterococcus* indicator microorganisms (Table II). The sandwich technique demonstrated the lack of bacteriophages, which would have prevented the antagonistic action against the indicator microorganisms.

The antagonistic substances produced by 45 of the 53 *Lactobacillus* isolates were inactivated after treatment with the 4 proteolytic

**Table II. Results of analysis of variance of the inhibition of indicator microorganisms by the *Lactobacillus* isolates**

| Species of isolate | Indicator microorganisms; mean diameter of inhibitory zone (and standard deviation), mm[a] | | |
|---|---|---|---|
| | *Enterococcus* | *Listeria* | *Salmonella* |
| *L. reuteri* (*n* = 30) | 1.48 (0.54) Aa | 2.68 (1.80) Ba | 2.71 (1.37) Ba |
| *L. salivarius* (*n* = 12) | 2.02 (0.29) Aab | 2.73 (0.59) Aa | 2.55 (1.49) Aa |
| *Lactobacillus* spp. (*n* = 11) | 2.50 (1.23) Ab | 2.98 (0.85) Aa | 2.38 (0.61) Aa |

[a] Capital letters compare the sensitivity of the indicator microorganisms to the antagonistic substances produced by each *Lactobacillus* isolate (rows). Lower-case letters compare the inhibitory effect of the antagonistic substances on each indicator organism (columns). Mean diameters followed by the same letter did not differ significantly ($P > 0.05$).

**Table III. Sensitivity to protease enzymes of the antagonistic substances produced by the *Lactobacillus* isolates**

| Enzyme | Sensitivity,[a] AU/mL (and no. of isolates sensitive or resistant) | | |
|---|---|---|---|
| | *L. reuteri* | *L. salivarius* | *Lactobacillus* spp. |
| Pronase E | 0.0 (30) | 0.0 (12) | 0.0 (11) |
| α-chymotrypsin | 0.0 (30) | 0.0 (12) | 0.0 (11) |
| Trypsin | 0.0 (30) | 0.8 (1) | 0.0 (11) |
| Pepsin | 0.4 (1) | 0.0 (12) | 0.0 (11) |
| | 0.8 (5) | | |
| | 1.6 (1) | | |
| Controls[b] | 0.2 (2) | 0.4 (1) | 0.4 (1) |
| | 0.4 (5) | 0.8 (7) | 0.8 (4) |
| | 0.8 (12) | 1.6 (4) | 1.6 (6) |
| | 1.6 (11) | | |

[a] Defined as the reciprocal of the highest dilution causing inhibition of the indicator strain multiplied by 100 to express the results as activity units per milliliter.

[b] Solutions of cell-free supernatant in 0.1 M sodium phosphate buffer without enzymes.

enzymes (Table III), which indicates the proteic nature of the substances. All the isolates were sensitive to pronase E and α-chymotrypsin, whereas 7 (23%) of the *L. reuteri* isolates were resistant to pepsin, and 1 *L. salivarius* isolate was resistant to trypsin.

## Discussion

From the original 474 bacterial isolates of *Lactobacillus* spp., we identified 30 of *L. reuteri*, 12 of *L. salivarius*, and 11 of *Lactobacillus* spp. that had antagonistic activity against gram-positive and gram-negative microorganisms. The inhibition by proteases of this activity in 85% of these isolates suggested that the antagonistic substances produced by the *Lactobacillus* in this study could be antimicrobial peptides or bacteriocins.

In poultry production, *Lactobacillus* species are used as a probiotic to produce a variety of antimicrobial substances (21) that inhibit gram-positive and gram-negative bacteria (22). Some bacteriocins originating from *Lactobacillus*, such as acidophilin and lactocidin, exhibit a broad spectrum of action against numerous genera of pathogenic and nonpathogenic bacteria (23).

The efficacy and spectrum of action of lactic acid bacteria against pathogenic microorganisms are based on the action of bacteriocins and a combination of antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriophages (24). The antagonistic methods used in the present study, spot-on-the-lawn and well diffusion, were effective in demonstrating the sensitivity of the indicator strains; however, the size of the bacterial inhibition zone varied according to the antagonistic method used. The activity of a bacteriocin can be estimated by the size of the inhibition zones produced in a diffusion test (12). In the present study, from the diameter of the inhibition zone we determined that the *Salmonella* indicator microorganisms were more sensitive than the *Enterococcus* indicator microorganisms to the antagonistic substances produced by *L. reuteri*. However, the *Lactobacillus* spp. isolates had a greater effect on the *Enterococcus* indicator microorganisms than did *L. reuteri*. Some *Lactobacillus* isolates were antagonistic to indicator microorganisms of the same genus (*L. delbrueckii*, *L. casei*, *L. fermentum*, and *L. helveticus*) by the spot-on-the-lawn method but not the well-diffusion method.

By use of the well-diffusion simultaneous antagonism method with anaerobic incubation, the action of hydrogen peroxide was precluded, demonstrating that the inhibition zones formed by the supernatants against the *Enterococcus*, *Listeria*, and *Salmonella* indicator microorganisms were due to proteic action specifically. The action of organic acids could also be precluded since modified MRS culture medium supplemented with only 0.05% glucose and adjusted to pH 6 was used, thus reducing fermentation and, consequently, the production of organic acids. The use of the sandwich technique prevented the diffusion of bacteriophages. Finally, the loss of inhibitory capacity of most of the *Lactobacillus* isolates after treatment with proteolytic enzymes indicated their proteic nature.

In conclusion, *Lactobacillus* species used as probiotic bacteria produce a variety of antimicrobial substances, which are mainly of proteic origin, called bacteriocins. Those produced by lactic acid bacteria demand particular attention because of their potential application to the food industry as natural antimicrobial means of food preservation and as probiotics for use in poultry production.

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